JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2010, p. 3666–3674 Vol. 48, No. 10
0095-1137/10/$12.00 doi:10.1128/JCM.00866-10

Cholera between 1991 and 1997 in Mexico Was Associated with Infection by Classical, El Tor, and El Tor Variants of *Vibrio cholerae*[▽]

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Received 29 April 2010/Returned for modification 9 June 2010/Accepted 17 July 2010

***Vibrio cholerae* O1 biotype El Tor (ET), the cause of the current 7th pandemic, has recently been replaced in Asia and Africa by an altered ET biotype possessing cholera toxin (CTX) of the classical (CL) biotype that originally caused the first six pandemics before becoming extinct in the 1980s. Until recently, the ET prototype was the biotype circulating in Peru; a detailed understanding of the evolutionary trend of *V. cholerae* causing endemic cholera in Latin America is lacking. The present retrospective microbiological, molecular, and phylogenetic study of *V. cholerae* isolates recovered in Mexico ($n = 91$; 1983 to 1997) shows the existence of the pre-1991 CL biotype and the ET and CL biotypes together with the altered ET biotype in both epidemic and endemic cholera between 1991 and 1997. According to sero- and biotyping data, the altered ET, which has shown predominance in Mexico since 1991, emerged locally from ET and CL progenitors that were found coexisting until 1997. In Latin America, ET and CL variants shared a variable number of phenotypic markers, while the altered ET strains had genes encoding the CL CTX (CTX^{CL}) prophage, $ctxB^{CL}$ and $rstR^{CL}$, in addition to resident $rstR^{ET}$, as the underlying regional signature. The distinct regional fingerprints for ET in Mexico and Peru and their divergence from ET in Asia and Africa, as confirmed by subclustering patterns in a pulsed-field gel electrophoresis (NotI)-based dendrogram, suggest that the Mexico epidemic in 1991 may have been a local event and not an extension of the epidemics occurring in Asia and South America. Finally, the CL biotype reservoir in Mexico is unprecedented and must have contributed to the changing epidemiology of global cholera in ways that need to be understood.**

In 1991, when cholera reemerged after being absent from Latin America for about a century, millions of people were affected, with nearly 9,000 dying in 1993 alone (13). Following its first appearance along the coast of Peru in January 1991, cholera rapidly spread to all countries in Latin America except Uruguay, reaching Mexico in June of the same year (13, 19, 25, 32). Since then, there has been a great interest in understanding the source and transmission of cholera in Latin America. Limited genetic analysis of *Vibrio cholerae* O1 biotype El Tor (ET) strains identified from the epidemic showed that they have a unique signature, distinguishing them from 7th pandemic ET strains (25). However, later studies showed the clonal nature of the bacterium, suggesting that the Latin American epidemic was an extension of the 7th pandemic caused by ET strains from the Western Hemisphere (34).

V. cholerae O1 and O139 are the two serogroups known to cause cholera. *V. cholerae* O1 has two biotypes, classical (CL) and ET, which differ in specific phenotypic traits (hemolysis of sheep erythrocytes, agglutination of chicken erythrocytes, sensitivity to polymyxin B [PMB], phage susceptibilities, and Voges-Proskauer [VP] test results) (15) and genotypic traits (*ctxB*, *acfB*, *tcpA*, and *rstR*). In addition, the CL and ET biotype strains differ in two major genomic regions, namely, the Vibrio seventh pandemic pathogenicity island I (VSP-I) and VSP-II, that are unique to the 7th pandemic ET biotype (10). Biotype CL caused the first six of the seven cholera pandemics recorded between 1817 and 1923 (26), with five of these affecting the American continents. Ever since the first cases were detected in the Americas in the 1830s, endemic cholera continued to be prevalent until 1895 (18).

Historically, cholera has been endemic in Asia for centuries (26), with Asia being at the center of each of the seven cholera pandemics (10, 26). Although the ET biotype was first reported in 1905 and the 6th pandemic, caused by the CL biotype, lasted until 1923, it was not until the early 1960s that the ET biotype displaced the CL biotype in Asia and became the causal agent of the 7th pandemic (33). The CL biotype maintained a low profile in its last recognized niche in the coastal ecosystem of the Bay of Bengal, before it disappeared as a causal agent of clinical disease in the 1980s (33).

Over the past few years, the ET biotype causing Asiatic cholera has shown remarkable changes in its phenotypic and genetic characteristics (23). Recent molecular analysis of ET strains causing acute watery diarrhea in Bangladesh shows them to be hybrids because they possess phenotypic and ge-

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▽ Published ahead of print on 28 July 2010.

TABLE 1. Phenotypic and related genetic characteristics of *V. cholerae* O1 biotypes ET and CL and hybrid variants of both biotypes isolated in Mexico from 1983 to 1997[a]

Yr of isolation or strain	No. of strains	Source	Serotype	Phenotypic properties				Genetic screening by PCR					Deduced biotype
				CCA	Sensitivity			*ctxA*	*ctxB* type[b]	*tcpA*	*rtxC*	*rstR* type	
					PMB (50 U)	CL-specific phage IV	ET-specific phage V						
1983	1	Clin	Inaba	−	S	S	R	+	CL	CL	−	CL	CL
1991	6	Clin	Inaba	+	R	R	S	+	ET	ET	ET	ET	ET
1991	2	Clin	Ogawa	+	R	R	S	+	CL	ET	ET	ET, CL	Alt-ET
1991	19	Clin	Ogawa	−	R	R	S	+	CL	ET	ET	ET, CL	Alt-ET
1991	2	Clin	Ogawa	−	S	R	S	+	CL	ET	ET	ET, CL	Alt-ET
1992	3	Clin	Inaba	+	R	R	S	+	ET	ET	ET	ET	ET
1992	1	Env	Inaba	+	S	R	S	+	ET	ET	ET	ET	ET
1992	1	Clin	Inaba	+	R	R	R	+	ET	ET	ET	ET	ET
1992	1[c]	Env	Inaba	−	S	R	R	−	−	CL	−	CL	CL
1993	6	Clin	Ogawa	−	R	R	S	+	CL	ET	ET	ET, CL	Alt-ET
1993	1	Clin	Ogawa	+	S	R	S	+	ET	ET	ET		ET
1993	1	Clin	Ogawa	−	S	R	S	+	CL	ET	ET	ET, CL	Alt-ET
1994	1	Clin	Inaba	+	R	R	S	+	ET	ET	ET		ET
1994	1	Env	Inaba	+	R	R	S	+	CL	ET	ET	ET, CL	Alt-ET
1995	1	Clin	Ogawa	−	S	R	R	+	CL	CL	−	CL	CL
1995	1	Clin	Inaba	+	R	R	S	+	ET	ET	ET	ET	ET
1995	1	Env	Inaba	−	R	R	R	+	CL	CL	−	CL	CL
1995	1	Clin	Inaba	−	R	R	S	+	CL	ET	ET	ET, CL	Alt-ET
1995	2	Clin	Ogawa	−	R	R	S	+	CL	ET	ET	ET, CL	Alt-ET
1995	1	Env	Ogawa	−	R	R	R	+	CL	ET	ET	ET, CL	Alt-ET
1995	1	Clin	Inaba	−	S	R	S	+	CL	ET	ET	ET, CL	Alt-ET
1997	8	Clin	Ogawa	+	R	R	S	+	CL	ET	ET	ET, CL	Alt-ET
1997	26	Clin	Ogawa	−	R	R	S	+	CL	ET	ET	ET, CL	Alt-ET
1997	1	Env	Inaba	−	S	S	R	+	CL	CL	−	CL	CL
1997	1	Clin	Inaba	−	S	S	R	+	CL	CL	−	CL	CL
1997	1	Env	Inaba	−	R	R	S	+	CL	ET	ET	ET, CL	Alt-ET
N16961		Control	Inaba	+	R	R	S	+	ET	ET	ET	ET	El Tor
O395		Control	Ogawa	−	S	S	R	+	CL	CL	−	CL	CL

[a] A total of 91 strains were evaluated. Abbreviations: Clin, clinical; Env, environmental; R, resistant; S, sensitive; Alt, altered.
[b] Determined by MAMA-PCR (21).
[c] Serologically nonreactive to antibodies specific for O1 or O139.

notypic traits of the CL biotype against an ET background (23). Subsequent retrospective studies showed that all of the O1 ET strains isolated in Bangladesh since 2001 have been hybrids of both the CL and ET biotypes, while those isolated before 2001 contained all the attributes of the 7th pandemic *V. cholerae* O1 ET strains (22). Such genetic changes among ET strains causing cholera in Latin America were also evident from the changing serotypes, electrophoretic types, ribotypes, and pulsed-field gel electrophoresis (PFGE) types (3, 9, 11, 27). While the ET prototype has been completely replaced by an altered ET in Asia and Africa (29), recent data show the nature of the ET prototype (7th pandemic) of *V. cholerae* O1 isolated in Peru between 1991 and 2003 (24). The Peruvian ET strains that seem to be closely linked clonally to the Asian and African ET prototype strains were shown to have a distinct region in VSP-II that differentiates them from the ET prototype strains isolated in other continents (24). To better understand the dynamics of the cholera epidemic in Latin America, the present study analyzed 91 *V. cholerae* O1 strains isolated from both clinical and environmental sources in Mexico between 1983 and 1997. *V. cholerae* O1 ET strains from Peru (1991 to 1999), Bangladesh (1985 to 2007), and Zambia (1996 to 2004) were also included for comparative purposes.

MATERIALS AND METHODS

Bacterial strains. The *V. cholerae* serogroup O1 strains characterized and compared in the present study are shown in Table 1, together with their sources and years of isolation.

Confirmation of *V. cholerae* strain types. The Latin American isolates of *V. cholerae* used in the present study were obtained from the Department of Public Health, Faculty of Medicine, National Autonomous University of Mexico (UNAM). These strains were of patient and surface water origin and were isolated as part of the nationwide cholera surveillance conducted between 1983 and 1997 (3). The bacterial strains were shipped in soft agar, their identities were confirmed by standard culture methods, and their serogroups and biotypes were identified by a combination of biochemical and molecular methods, as described previously (1).

Serogrouping. The serogroups of the *V. cholerae* isolates that were identified using biochemical and molecular methods were confirmed serologically by a slide agglutination test using specific polyvalent antisera to *V. cholerae* O1 and O139, followed by a monoclonal antibody that is specific for both serogroups (1).

Biotyping. Biotyping involved a number of phenotypic tests: chicken erythrocyte agglutination, sensitivity to polymyxin B, and Mukerjee CL phage IV and Mukerjee ET phage V tests (15). To complement the biotype characterization by phenotypic traits, PCR assays were carried out using previously described procedures that were targeted to detect *tcpA* (CL and ET variants) and the type of the *rstR* gene encoding the phage transcriptional regulator (16).

Storage of strains. *V. cholerae* strains whose types were confirmed by biochemical, serological, and molecular methods were subcultured on gelatin agar (GA) plates; and a single representative colony from the GA was aseptically inoculated into T1N1 broth (1% Trypticase, 1% NaCl), incubated at 37°C for 3 to 4 h, and stored at −80°C with 15% glycerol until it was required.

Genomic DNA preparation. Genomic DNA extraction was carried out following previously described methods (24).

Confirmation of serogrouping by PCR assays. The subtypes of all the strains were reconfirmed using a *V. cholerae* species-specific *ompW* PCR (24). The serogroups of these strains were reconfirmed using polyvalent O1 and monovalent Inaba and Ogawa antisera and by multiplex PCR targeted to identify genes encoding O1 (*wbe*) and O139 (*wbf*)-specific O biosynthetic genes and the cholera toxin (CTX) gene (*ctxA*) (14).

MAMA-PCR for determination of *ctxB* gene type. The mismatch amplification mutation assay (MAMA) was recently developed to detect the sequence polymorphism between the CL and ET *ctxB* genes ($ctxB^{CL}$ and $ctxB^{ET}$, respectively) by focusing on nucleotide position 203 of the *ctxB* gene (21). MAMA-PCR was performed to test for the presence of the *ctxB* genes specific for the CL and ET biotypes. A conserved forward primer (Fw-con, 5′-ACTATCTTCAGCATATG CACATGG-3′) and two allele-specific polymorphism detection primers, Rv-cla (5′-CCTGGTACTTCTACTTGAAACG-3′) and Rv-elt (5′-CCTGGTACTTCT ACTTGAAACA-3′), were used. PCR conditions were as follows: after initial denaturation at 96°C for 2 min, 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 10 s, extension at 72°C for 30 s, and a final extension at 72°C for 2 min. The resulting *V. cholerae* O1 isolates, O395 CL and N16961 ET, were used as standard reference strains.

PFGE. The whole agarose-embedded genomic DNA from *V. cholerae* was prepared. PFGE was carried out with a contour-clamped homogeneous electrical field (CHEF-DRII) apparatus (Bio-Rad), according to procedures described elsewhere (6). The conditions used for separation were as follows: 2 to 10 s for 13 h, followed by 20 to 25 s for 6 h. An electrical field of 6 V/cm was applied at an included field angle of 120°. Genomic DNAs of the test strains were digested by the NotI restriction enzyme (Gibco-BRL, Gaithersburg, MD), and *Salmonella enterica* serovar Braenderup was digested by XbaI, with the fragments being used as molecular size markers. The restriction fragments were separated in 1% pulsed-field-certified agarose in 0.5× TBE (Tris-borate-EDTA) buffer. In the postelectrophoresis gel treatment step, the gel was stained and destained. The DNA was visualized using a UV transilluminator, and images were digitized via a one-dimensional gel documentation system (Bio-Rad).

Image analysis. The fingerprint pattern in the gel was analyzed using a computer software package, Bionumeric (Applied Maths, Belgium). After background subtraction and gel normalization, the fingerprint patterns were subjected to typing on the basis of banding similarity and dissimilarity using Dice similarity coefficient and unweighted-pair group method using average linkages (UPGMA) clustering methods, as recommended by the manufacturer; these were graphically represented as dendrograms.

RESULTS

Microbiological and serological tests. All tested strains (n = 91) produced characteristic colonies typical of *V. cholerae* when they were grown on taurocholate tellurite gelatin agar (TTGA). The resulting colonies gave biochemical reactions typical of *V. cholerae*, and all except one strain reacted to the monoclonal antibody specific for O1 but not to that for O139. All *V. cholerae* O1 strains, including the serologically nonreacting strain, reacted to monovalent Inaba and Ogawa antisera, suggesting that all belonged to serogroup O1.

Amplification of primers specific for *V. cholerae* serogroup O1 and *ctxA* by PCR assays. All strains (n = 91) amplified the primers for the *V. cholerae* species-specific gene *ompW*, and all except the strain serologically determined to be non-O1/O139 amplified the primers specific for the O biosynthesis gene *wbe* but not those specific for *wbf*. In addition, all the strains serologically confirmed to be O1 amplified the primers for the cholera toxin gene *ctxA*, confirming that all strains were *ctx*-positive *V. cholerae* O1.

Phenotypic and related genotypic characteristics. The results of the major phenotypic and related genetic characterizations of the *V. cholerae* O1 strains (n = 91) are presented in Table 1. The *V. cholerae* O1 strains varied greatly in their phenotypic and related genetic characteristics. Twenty-two of the *V. cholerae* strains showing biotype ET-specific phenotypic traits, such as chicken cell agglutination (CCA), sensitivity to ET-specific phage V, and resistance to both PMB and CL-specific phage IV, were primarily recognized to be biotype ET (Table 1). Three of the CCA-negative strains matched biotype CL reference strain O395 in being sensitive to both PMB and CL-specific phage IV but not to ET-specific phage V, thus being recognized primarily as biotype CL (Table 1). Most of the *V. cholerae* strains were phenotypic variants of ET because they had major ET traits but failed to show all the properties typical of the ET reference strain, N16961 (Table 1).

Although *V. cholerae* O1 strains in Mexico varied significantly in their biotype-specific, phenotypic markers, 85 of the 91 phenotypically biotype ET strains amplified the primers for the *rtxC* gene, an ET-specific marker, confirming their ET trait. Fourteen of the strains that were primarily identified to be ET amplified the primers for the $tcpA^{ET}$ and $rstR^{ET}$ genes but not for the $tcpA^{CL}$ and $rstR^{CL}$ genes, further confirming their typical ET identity (Table 1). The remaining 71 ET strains were variants, since they possessed various numbers of major phenotypic markers for the CL biotype, in addition to having major ET traits and amplifying the primers for the ET-specific alleles of the $tcpA^{ET}$ and $rstR^{ET}$ genes (Table 1). All of these ET variants also amplified the gene encoding the CTX prophage, $rstR^{CL}$, in addition to amplifying the resident $rstR2^{ET}$, and were primarily designated altered ET.

In sharp contrast, the template DNA of the six remaining *V. cholerae* strains, most of which varied in their major phenotypic traits, including the one that was not recognized by O1- or O139-specific antibodies but that reacted to Inaba antisera, did not amplify the primers for biotype ET-specific marker gene *rtxC*, suggesting possible CL attributes (Table 1). Of these, the five serologically O1 strains amplified the primers for the $tcpA^{CL}$ and $rstR^{CL}$ genes but not those for the $tcpA^{ET}$ and $rstR^{ET}$ genes. This confirms that these five strains, one of which was a pre-1991 strain isolated in Mexico, belong to the CL biotype, which disappeared as a cause of cholera in the 1980s. The serologically non-O1/O139 strain, which possessed certain CL attributes, such as being sensitive to both CCA and PMB, despite being resistant to both CL- and ET-specific phages, amplified the primers for the biotype-specific gene $tcpA^{CL}$ allele but not for the $tcpA^{ET}$ allele or any of the primers for the gene encoding CTX phage, *ctxA* or *rstR*. These results suggest that the serologically non-O1/O139 strain may have originally been toxigenic *V. cholerae* O1 and also biotype CL but had its CTX prophage excised.

MAMA-PCR. MAMA-PCR using primers specific for the CL or ET biotype offers a precise and accurate method for determining the type of CTX that *V. cholerae* produces. The *V. cholerae* O1 strains, including the CL (O395) and ET (N16961) reference strains, were analyzed to determine their CTX type by MAMA-PCR. As shown in Table 1, *V. cholerae* O1 strains that were primarily identified to be ET amplified the ET-specific $ctxB^{ET}$ gene but not the $ctxB^{CL}$ gene, confirming their prototype ET traits. In contrast, all of the ET strains possessing the gene encoding CTX^{CL} phage, $rstR^{CL}$, in other words, the altered ET strains, including the five CL biotype strains, amplified the $ctxB^{CL}$ gene but not the $ctxB^{ET}$ gene, again confirming their CTX to be that of the CL biotype. However, the

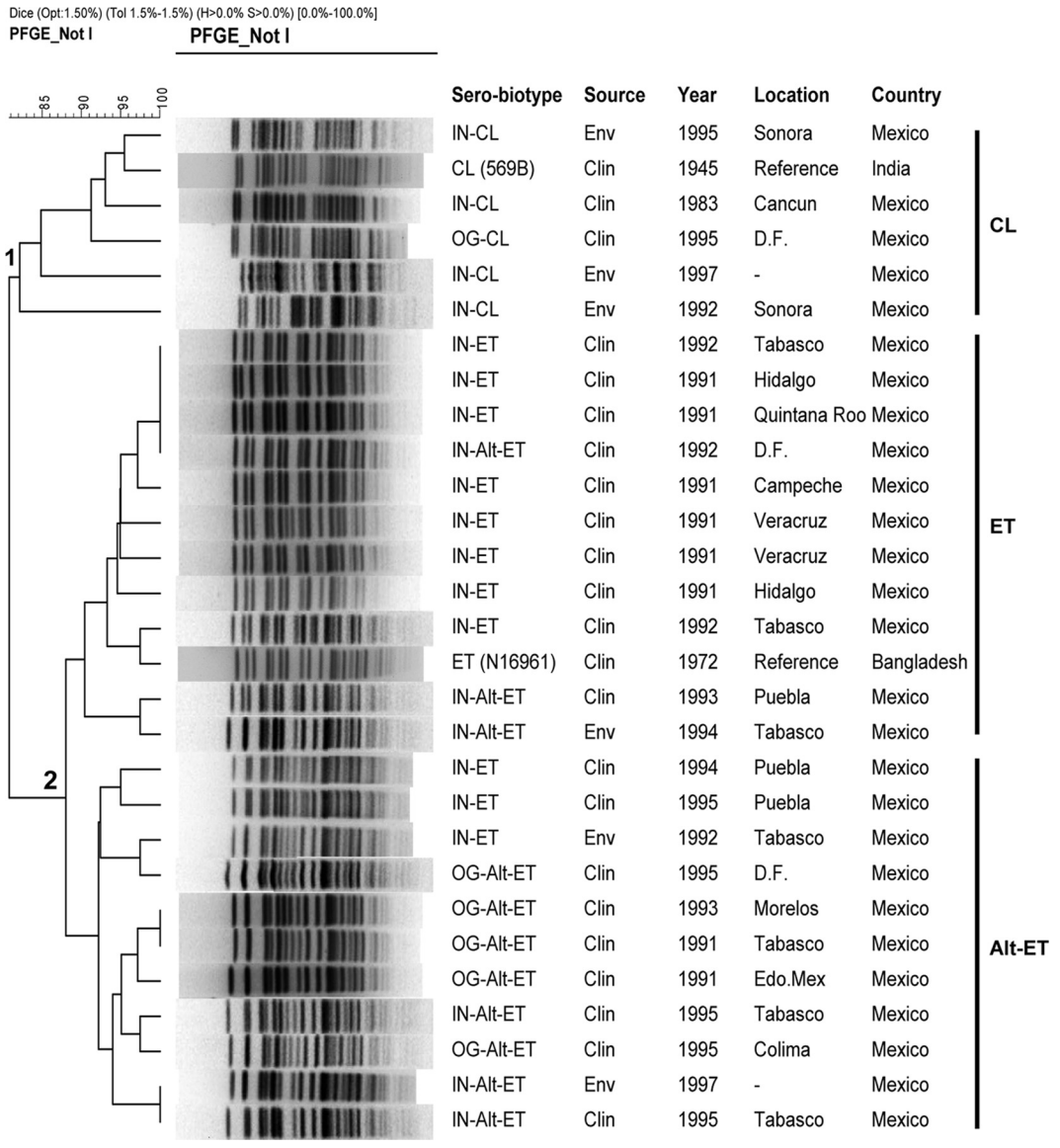


FIG. 1. Dendrogram showing genomic fingerprints of *V. cholerae* O1 strains isolated in Mexico (1983 to 1997), North America. The dendrogram was prepared by Dice similarity coefficient and UPGMA clustering using the PFGE patterns of NotI-digested genomic DNA. The scale bar at the top shows the correlation coefficient (%). Two major clusters separating the CL biotype from the ET biotype strains show the respective lineages. The CL cluster, which includes the North American CL strains with the CL reference control strain (strain 569B), shows high degrees of divergence among the strains. The major ET cluster shows two subclusters that separate the prototype ET (Pro-ET) (including reference control strain N16961) from the altered ET (Alt-ET) strains, suggesting two different lineages for them. IN, Inaba; OG, Ogawa; Clin, clinical; Env, environmental; Edo., estado (state).

serologically non-O1/O139 strain failed to amplify any of the two biotype-specific alleles by MAMA-PCR.

The altered ET that has been confirmed in Mexico along with the progenitor ET and CL biotype strains presented direct evidence of a genetic transition from the ET prototype to the altered ET. Of the seven environmental strains analyzed in the present study, three, including the serologically nonreacting strain, were identified to be of the CL biotype, one was identified to be of the ET biotype, and the remaining three were identified to be of the altered ET biotype (Table 1), suggesting an aquatic reservoir for them.

PFGE and cluster analysis. The NotI-digested genomic DNAs of both clinical and environmental *V. cholerae* O1 strains of different spatiotemporal origins and the resulting different biotype categorizations, including one pre-1991 CL strain isolated in Mexico in 1983, were subjected to PFGE to determine their genetic relatedness and clonal origin. The NotI restriction enzyme digested the genomic DNA of both the test and the reference control strains into 20 to 23 fragments (Fig. 1); the molecular sizes of the DNA fragments ranged from 20 to 350 kb. *V. cholerae* O1 strains of the ET and CL biotypes presented overall banding patterns that were charac-

teristic of their respective ET and CL reference controls (Fig. 1). The serologically non-O1 but genotypically confirmed O1 CL strain had the signature banding pattern of the CL reference control, suggesting a CL ancestry.

Cluster analysis, which was performed with dendrograms (prepared by Dice similarity coefficient and UPGMA clustering methods) obtained from the PFGE patterns of NotI-digested genomic DNA, separated both the CL biotype strains (cluster A) from the ET biotype strains (cluster B) and the altered ET biotype strains from the progenitor ET biotype strains, irrespective of their source (Fig. 1). This analysis further confirmed the biotype categorization of *V. cholerae* O1 strains made in the present study, thereby indicating their genetic fingerprints. A great deal of variation was observed in the clustering patterns of the genetically patchy strains belonging to the two biotypes. Subclustering was identified in the major biotype-specific clusters, which separated the prototype CL strains from their variants and the progenitor ET (cluster B1) from the altered ET (cluster B2), suggesting that each followed a different lineage. Both clinical and environmental strains of a particular biotype category clustered together, suggesting that they are clonally related.

To further understand the clonal link between the *V. cholerae* O1 strains occurring in Latin America, a separate cluster analysis was performed using the PFGE (NotI) images of the genomic DNA of O1 strains isolated in Mexico, together with those of selected ET strains causing endemic cholera in Peru (1991 to 1999). As shown in Fig. 2, two major biotype-specific clusters separated the CL biotype strains (cluster A) from the ET biotype strains (cluster B), irrespective of their spatiotemporal origin, suggesting biotype-specific lineages for them. Subclustering was observed under the major ET cluster (cluster B), which separated the Peruvian ET strains (cluster B1) from the prototype ET strains (cluster B2) and the altered ET strains (cluster B3) of Mexico, showing divergence and regional signatures.

Since the ET prototype causing the 1991 epidemic in Mexico showed a regional signature different from that of the ET biotype that caused the Peruvian epidemic at the same time, cluster analysis was performed by dendrogram analysis with the PFGE images of the *V. cholerae* O1 strains obtained in the present study to compare them with those of the CL and ET (altered ET) strains of Asia and Africa. Again, two major clusters (Fig. 3) separated the CL strains (cluster A) from the ET strains (cluster B), irrespective of their origin. However, a great deal of divergence between the strains of the two biotypes was observed, as seen in the similarity indices. Despite being divergent, the pre- and post-1991 prototype CL strains isolated in Mexico clustered together with the Asian CL strains (cluster A), suggesting the same lineage for all of these strains. The ET cluster (cluster B), which also displayed considerable heterogeneity, underwent further subclustering that separated the altered ET strains of Mexico (cluster B1) from those of Asia and Africa (cluster B4) and that separated the ET prototype strain of Mexico (cluster B2) from that of Peru (cluster B3), suggesting type-specific lineages and regional signatures for *V. cholerae* O1.

DISCUSSION

The 1991 epidemic in Peru was shown to be a clonal expansion of the 7th ET biotype pandemic currently being seen in Asia (3, 5, 20). A significant and recent development has been the emergence of an altered ET possessing the gene encoding the CTX that is specific for the CL biotype. This is interesting, because the CL biotype caused the first six pandemics before being replaced by the ET biotype, and therefore, the CL biotype was considered to be extinct or to have at least disappeared as a cause of clinical disease. While the altered ET has already replaced the prototype 7th pandemic ET in Asia (11) and Africa (17, 21), according to a recent study, the ET causing endemic cholera in Peru up until 2003 was of the 7th pandemic ET prototype (18). The present retrospective study of the phenotypic, molecular, and phylogenetic characterizations of *V. cholerae* O1 isolated in Mexico shows that the CL biotype was circulating in Mexico before and after 1991. The study also reveals that both the ET and CL biotypes, together with their hybrid variants, were consistently present in the aquatic environment and were the causal agents for the 1991 epidemic and the subsequent endemic cholera in Mexico.

The results of the microbiological culture and the biochemical and serological tests primarily confirmed that the *V. cholerae* isolates in Mexico were serogroup O1, while one *V. cholerae* strain did not react serologically to antiserum specific for O1 or O139. These primary microbiological results were further complemented by simplex and multiplex PCR assays, which included *V. cholerae* species-specific gene (*ompW*) *ctxA* (encoding subunit A of CTX) and the gene encoding serogroup O1 (*wbe*) (14), further confirming that the *V. cholerae* strain in Mexico was toxigenic and belonged to serogroup O1 (4, 19). The serologically nonreacting environmental strain that reacted to monovalent Inaba antiserum and that amplified the primers for the *ompW* and *wbe* genes (14) but not the primer for *ctxA* suggested that it was *ctx*-negative *V. cholerae* O1. The *ctx*-negative *V. cholerae* O1 strain, which occurs in aquatic environments, has been shown to arise following the loss of the CTX prophage (1).

V. cholerae O1 biotypes CL and ET can be distinguished by the differences in their phenotypic and genotypic characteristics (10, 15). The results of the phenotypic and genetic screening in the present study indicate that *V. cholerae* O1 strains circulating in Mexico included both CL and ET strains together with the variants of both biotypes. This finding is unprecedented, since it shows that the CL biotype was involved in endemic cholera caused by biotype ET at a time when the CL biotype was thought to be extinct (33). Furthermore, until recently, only strains of the ET prototype, such as those isolated in Peru, had been shown to be the cause of the Latin American epidemic, where the CL biotype has never been detected (4, 19, 34). Therefore, Mexico was clearly an important regional habitat for *V. cholerae* O1 strains that were different from those isolated in South America at the same time. The pre- and post-1991 CL biotype reservoir in Mexico must have contributed to the changing epidemiology of global cholera in ways that still need to be understood.

In the present study, the altered nature of the ET strains isolated in Mexico was confirmed, showing that their *ctxB* gene was of the CL biotype, the prototype of which was reported in

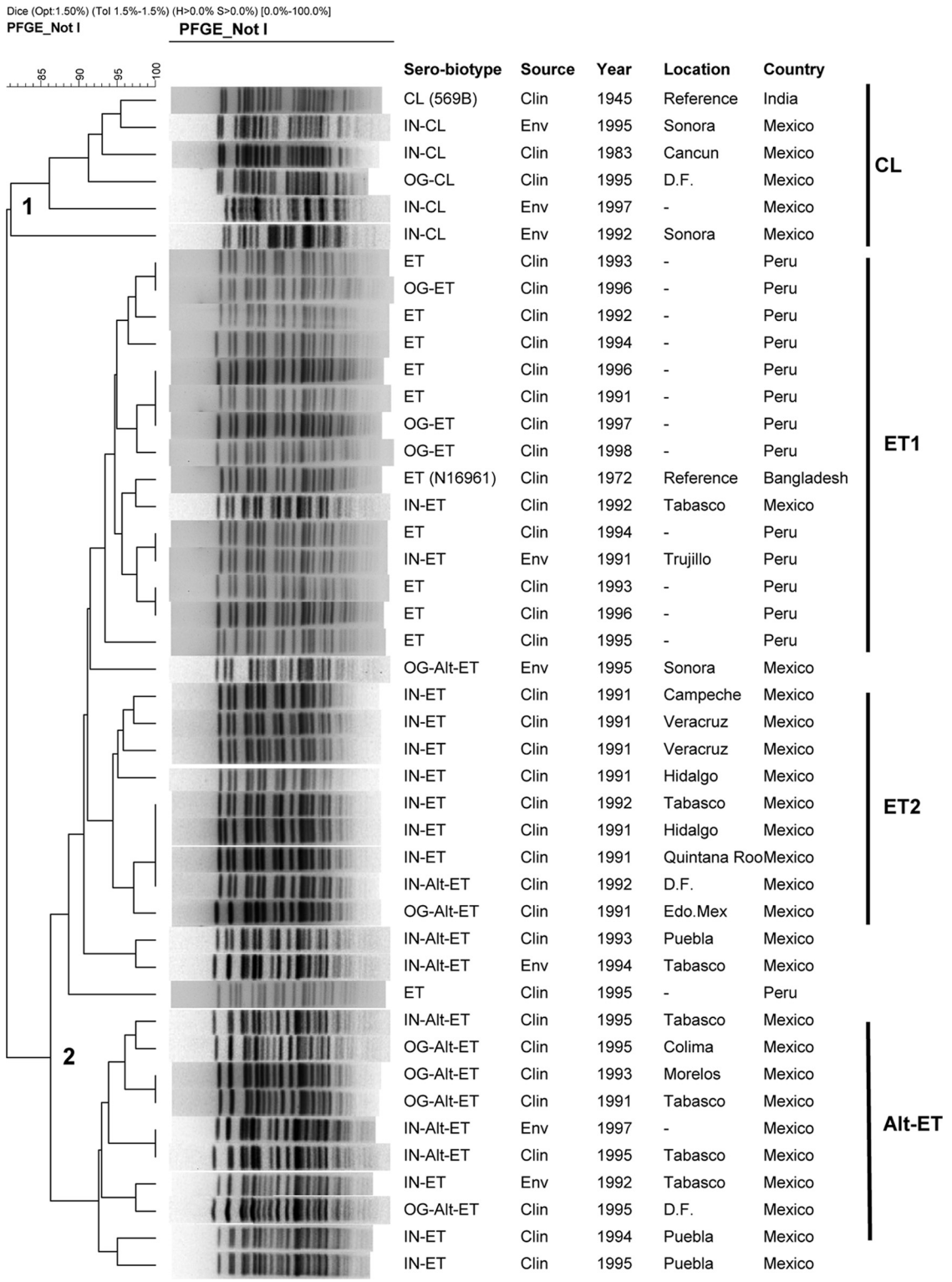
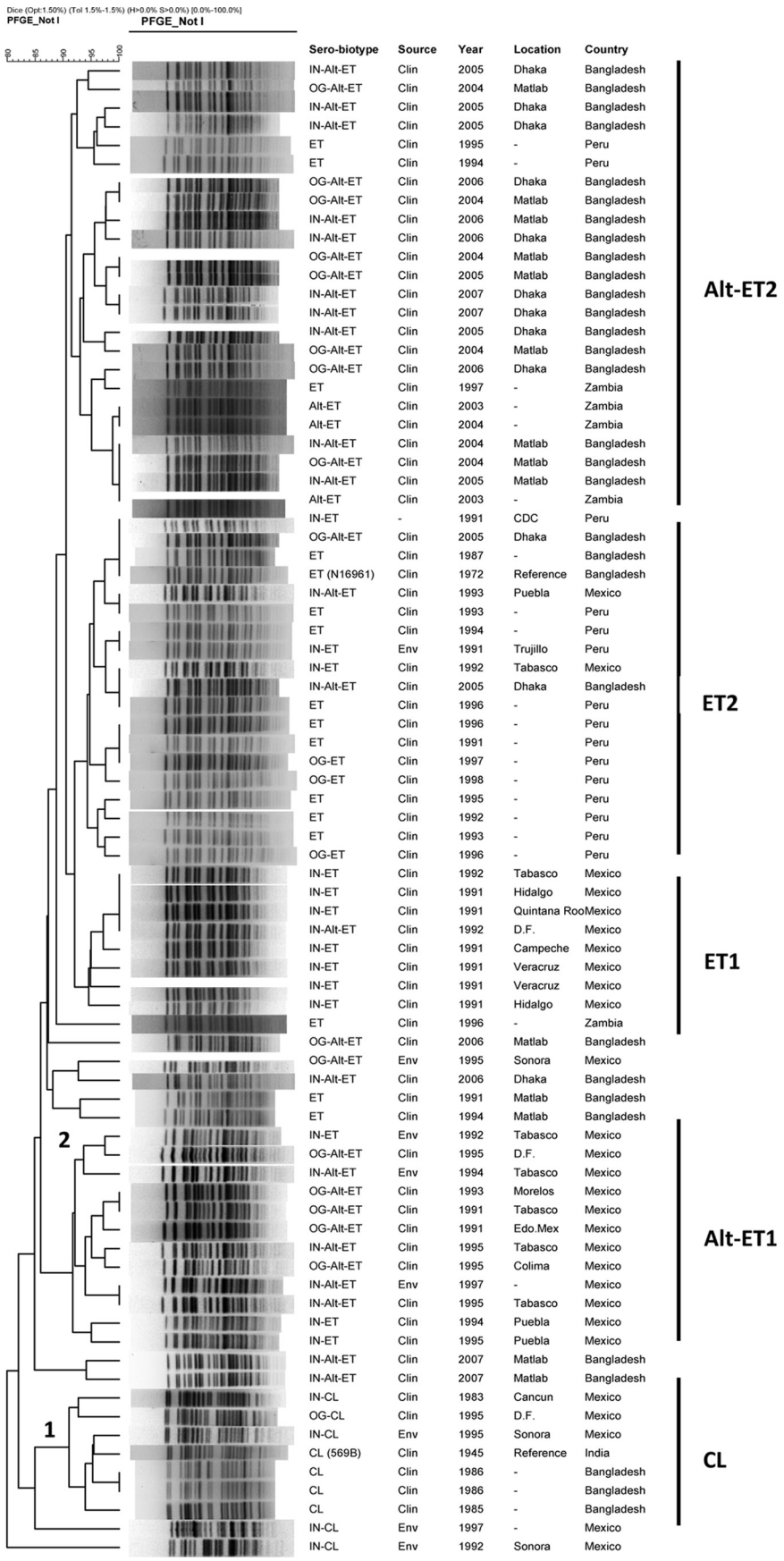


FIG. 2. Dendrogram showing genomic fingerprints of *V. cholerae* O1 isolated in Mexico (1983 to 1997) and Peru (1991 to 1998), Latin America. The dendrogram (prepared by Dice similarity coefficient and UPGMA clustering) was based on the PFGE (NotI) images of genomic DNA. The two major clusters, showing the degree of similarity (%), separated the CL from the ET biotype strains, suggesting respective biotype-specific lineages. The CL biotype cluster, which includes strains exhibiting signature PFGE patterns for CL reference control strain 569B, shows high degrees of divergence among the strains. The major ET cluster shows subclusters, separating the Peruvian prototype ET (ET1) subcluster from the rest while separating the Mexican prototype ET (ET2) subcluster from the Mexican altered ET (Alt-ET), suggesting regional and type-specific fingerprints. IN, Inaba; OG, Ogawa; Clin, clinical; Env, environmental; Edo., estado (state).


Dice (Opt:1.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE_Not I
PFGE_Not I
80
85
90
95
100
Sero-biotype Source Year Location Country
IN-Alt-ET Clin 2005 Dhaka Bangladesh
OG-Alt-ET Clin 2004 Matlab Bangladesh
IN-Alt-ET Clin 2005 Dhaka Bangladesh
IN-Alt-ET Clin 2005 Dhaka Bangladesh
ET Clin 1995 - Peru
ET Clin 1994 - Peru
OG-Alt-ET Clin 2006 Dhaka Bangladesh
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IN-Alt-ET Clin 2005 Dhaka Bangladesh
OG-Alt-ET Clin 2004 Matlab Bangladesh
OG-Alt-ET Clin 2006 Dhaka Bangladesh
ET Clin 1997 - Zambia
Alt-ET Clin 2003 - Zambia
Alt-ET Clin 2004 - Zambia
IN-Alt-ET Clin 2004 Matlab Bangladesh
OG-Alt-ET Clin 2004 Matlab Bangladesh
IN-Alt-ET Clin 2005 Matlab Bangladesh
Alt-ET Clin 2003 - Zambia
IN-ET - 1991 CDC Peru
OG-Alt-ET Clin 2005 Dhaka Bangladesh
ET Clin 1987 - Bangladesh
ET (N16961) Clin 1972 Reference Bangladesh
IN-Alt-ET Clin 1993 Puebla Mexico
ET Clin 1993 - Peru
ET Clin 1994 - Peru
IN-ET Env 1991 Trujillo Peru
IN-ET Clin 1992 Tabasco Mexico
IN-Alt-ET Clin 2005 Dhaka Bangladesh
ET Clin 1996 - Peru
ET Clin 1996 - Peru
ET Clin 1991 - Peru
OG-ET Clin 1997 - Peru
OG-ET Clin 1998 - Peru
ET Clin 1995 - Peru
ET Clin 1992 - Peru
ET Clin 1993 - Peru
OG-ET Clin 1996 - Peru
IN-ET Clin 1992 Tabasco Mexico
IN-ET Clin 1991 Hidalgo Mexico
IN-ET Clin 1991 Quintana Roo Mexico
IN-Alt-ET Clin 1992 D.F. Mexico
IN-ET Clin 1991 Campeche Mexico
IN-ET Clin 1991 Veracruz Mexico
IN-ET Clin 1991 Veracruz Mexico
IN-ET Clin 1991 Hidalgo Mexico
ET Clin 1996 - Zambia
OG-Alt-ET Clin 2006 Matlab Bangladesh
OG-Alt-ET Env 1995 Sonora Mexico
IN-Alt-ET Clin 2006 Dhaka Bangladesh
ET Clin 1991 Matlab Bangladesh
ET Clin 1994 Matlab Bangladesh
IN-ET Env 1992 Tabasco Mexico
OG-Alt-ET Clin 1995 D.F. Mexico
IN-Alt-ET Env 1994 Tabasco Mexico
OG-Alt-ET Clin 1993 Morelos Mexico
OG-Alt-ET Clin 1991 Tabasco Mexico
OG-Alt-ET Clin 1991 Edo.Mex Mexico
IN-Alt-ET Clin 1995 Tabasco Mexico
OG-Alt-ET Clin 1995 Colima Mexico
IN-Alt-ET Env 1997 - Mexico
IN-Alt-ET Clin 1995 Tabasco Mexico
IN-ET Clin 1994 Puebla Mexico
IN-ET Clin 1995 Puebla Mexico
IN-Alt-ET Clin 2007 Matlab Bangladesh
IN-Alt-ET Clin 2007 Matlab Bangladesh
IN-CL Clin 1983 Cancun Mexico
OG-CL Clin 1995 D.F. Mexico
IN-CL Env 1995 Sonora Mexico
CL (569B) Clin 1945 Reference India
CL Clin 1986 - Bangladesh
CL Clin 1986 - Bangladesh
CL Clin 1985 - Bangladesh
IN-CL Env 1997 - Mexico
IN-CL Env 1992 Sonora Mexico
Alt-ET2
ET2
ET1
Alt-ET1
CL
2
1

Bangladesh (22, 31). Therefore, the question is, why did the Peruvian *V. cholerae* O1 ET strains not switch from the prototype (24) to the altered type until recently, considering that the ET strains, such as those in Asia, Africa (2, 28, 29), and Mexico, did so in as early as 1991? The answer may not be precise, but it is likely that this change that took place in Mexico, but not in Peru (24), may have been prompted by the copresence of the CL and ET biotype strains in the aquatic environment, which was reported in Bangladesh up until the 1980s (33) but which was a phenomenon not seen in Peru (24).

There are two different possibilities for the emergence and global spread of the altered ET biotype in Asia and Africa (28, 29). The first one is that altered ET was the result of clonal expansion of a single ancestral ET that had acquired the *ctxB* gene of the CL biotype in one of the regions where cholera is endemic. The second one is that the emergence of the altered ET was not a single event but consisted of multiclonal events occurring independently in each region. Direct evidence supporting these hypotheses is lacking, however. A recent study has proposed that the transition took place between 1990 and 1994 for Southeast Asia, with all O1 ET strains isolated before 1990 being of the ET prototype (28). The present study is the first to provide direct evidence of the altered ET occurring with an array of other minor phenotypic variant strains of both biotypes together with the progenitor CL and ET strains that were involved in the 1991 epidemic and the subsequent endemic cholera in Mexico until 1997. The background epidemiological data and the patient selection criteria were not available for the clinical *V. cholerae* strains included in the present retrospective study; therefore, the possibility that the overrepresented altered ET (of the Ogawa serotype) strains in 1991 and 1997 were not disproportionately picked up from cases in well-defined time-space clusters cannot be ruled out. Nonetheless, the evidence of a genetic shift from prototype ET to altered ET in Latin America provided here suggests that this was a local event that occurred in Mexico and that was unrelated to what was happening in Asia and Africa at the time.

The hybrid variants of *V. cholerae* O1, designated Matlab variants, which were biotype ET but which had the phenotypic and genotypic traits of CL biotypes, were first reported from patients with acute secretory diarrhea in Matlab, Bangladesh (23), and then in Mozambique (2). Such genetic ET variants reported from Bangladesh had the $ctxB^{CL}$ gene in combination with either $rstR^{ET}$ only, $rstR^{CL}$ only, or both $rstR^{ET}$ and $rstR^{CL}$, irrespective of whether their *tcpA* alleles were of the CL or the ET biotype (30). The ET variants in Mozambique, on the other hand, had only the CTX^{CL} prophage-related genes, $ctxB^{CL}$ and $rstR^{CL}$, in pairs (2). The altered ET in the present study may be an analogue of the Matlab variant (23, 30) or Mozambique variants (2) of *V. cholerae* O1 ET strains, although the former differed from the variants in Asia (30) and Africa (2), with all the Latin American altered ET strains having $ctxB^{CL}$ and $rstR^{CL}$ genes, in addition to the resident $rstR^{ET}$ gene. Therefore, these results show the unique regional signature for the Latin American altered ET strains, further substantiating their independent emergence from the coexisting ET and CL biotype strains in Mexico.

Originally, the *V. cholerae* O1 ET that caused the 1991 epidemic in Latin America was shown to be homogeneous (4, 19, 27). It was only later that divergence in other serotypes was reported through the establishment of different electrophoretic types, ribotypes, and PFGE types (3, 9, 11, 22); and this has been corroborated more recently, with heterogeneity being reported among the ET strains isolated in Peru (24). Differences in the PFGE patterns of the CL and ET biotype strains and their variant strains were also observed in Mexico in the present study. Although the genetic basis for this divergence remains unknown, it may be due to CL and ET biotype strains sharing a niche in the aquatic ecosystem, as demonstrated in the present study, which allows interbiotype and cross-serotype genetic recombination, resulting in genetic reassortment (3, 9, 11).

V. cholerae O1 ET strains causing endemic cholera in Peru have been shown to be different from the 7th pandemic ET strains in Asia (24, 25). The subclustering in the PFGE (NotI)-based dendrogram in the present study, indicating the separation of the Mexican ET from the Peruvian ET and the Asian and African altered ET from the Latin American altered ET, further confirms the presence of specific regional signatures. These regional DNA fingerprints suggest that the 1991 epidemic in Mexico may have been a local event rather than an extension of the epidemics that occurred in Peru and Asia at that time (3, 19, 24, 25, 27, 34).

While the Latin American epidemic was considered to be imported via bilge water from ships coming from areas in Asia or Africa where cholera is endemic (20), sporadic but consistent cases of cholera on the Gulf Coasts of the United States and Mexico between 1965 and 1991 suggest a local source (4, 35). Following a cholera case that was reported in Cancun, Mexico, in 1983 (3), immediate studies failed to isolate any *V. cholerae* O1 strains, but diarrhea caused by non-O1 strains was shown to be highly prevalent (12). However, these results do not rule out a pre-1991 niche for *V. cholerae* in Latin America, as the serogroup O1 strains, which seldom respond to culturing media (7), can arise from non-O1 through seroconversion (5). This assumption may be further substantiated by confirmation of the *V. cholerae* O1 ET, CL, and altered ET biotypes, including the serologically non-O1 but phenotypically and genetically O1 CL variant isolated from the aquatic environments of Mexico in the present study, confirming their aquatic reservoir and

FIG. 3. Dendrogram showing genomic fingerprints of *V. cholerae* O1 isolates recovered in Mexico (North America) and Peru (South America) and their comparison with *V. cholerae* O1 isolates recovered in Bangladesh (Asia) and Zambia (Africa). Dendrogram (prepared by Dice similarity coefficient and UPGMA clustering) was based on the PFGE (NotI) patterns of genomic DNA. Two major clusters that separated the CL from the ET biotype strains, showing the degree of similarity (%), suggest biotype-specific lineages. The cluster CL includes the genetically divergent Latin American pre- and post-1991 CL strains with Asian CL strains and their reference control strain (strain 569B), suggesting the same clonal lineage for them. The major ET cluster divided the strains into four subclusters, separating the Mexican altered ET (Alt-ET1) strains from the altered ET of Asia and Africa (Alt-ET2) and Mexican prototype ET (ET1) strains from prototype ET (ET2) of Peru, depicting different signatures. IN, Inaba; OG, Ogawa; Clin, clinical; Env, environmental; Edo., estado (state).

showing that non-O1 serogroup strains can also arise from O1 serogroup strains. Therefore, even if the ecological niche for *V. cholerae* is not well defined for Latin America, the rapid spread of cholera in 1991 was presumably due to a preexisting, small population of plankton-bound O1 that exists mainly in coastal waters (4, 7, 35). The pre-1991 existence of the CL biotype, as confirmed in the present study, and its copresence with the prototype ET biotype and the phenotypic and genetic variants of both biotypes between 1991 and 1997 in Mexico may indicate a persistent niche for *V. cholerae* in Latin America.

The association of *V. cholerae* with plankton in the coastal waters of Peru and Mexico has been well documented (7, 8, 18). Although *V. cholerae* strains isolated in Mexico after 1997 were not available for analysis in the present study, a recent report shows the involvement of serogroup O1 in endemic cholera in the Gulf of Mexico coast after 1997 (17). Although this publication does not include the full genetic characteristics of *V. cholerae* O1 strains isolated after 1997, it does indicate a change in the ribosomal pattern that separates strains into two different groups before and after 1997. However, it would not be possible here to predict if the CL and ET progenitors that were found coexisting with the variant strains of both biotypes until 1997 still exist or were replaced by the altered ET that predominated in Mexico.

ACKNOWLEDGMENTS

This research was partially supported by NIID, Tokyo, Japan, and the ICDDR,B. The ICDDR,B is supported by donor countries and agencies, which provide unrestricted support to the center for its operation and research.

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